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<b>(54) Title:</b> METHOD FOR VITRIFICATION OF A BIOLOGICAL SPECIMEN  <b>(57) Abstract</b>  The present invention relates to a method of vitrification of a biological specimen. According to the method of the present invention, a biological specimen is directly exposed to a freezing material. Upon exposure to the freezing material, the biological specimen undergoes vitrification. The biological specimen which has undergone vitrification may be stored for a period of time, and then thawed at a later date. The thawed biological specimen remains viable. Preferred biological specimens according to the present invention are developmental cells.		

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## **METHOD FOR VITRIFICATION OF A BIOLOGICAL SPECIMEN**

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### **GOVERNMENT SUPPORT**

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10           Part of the work performed during development of this invention utilized U.S. Government Funds, specifically the National Institute of Child Health and Human Development, Grant No. HD22023. Therefore, the U.S. Government has certain rights in this invention.

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### **TECHNICAL FIELD**

          This invention relates to a method for vitrification of a biological specimen, such that the biological specimen remains viable after it is thawed.

## BACKGROUND OF THE INVENTION

The ability to cryopreserve oocytes, embryos, sperm and other similar biological specimens is critical to the widespread application of assisted reproductive technologies. However, due to the large volume of the cells and the high chilling sensitivity of oocytes and early embryos, cryopreservation techniques are not well developed in most species.

Traditionally, embryos are cryopreserved using "slow freezing techniques". Low concentrations of cryoprotectants and slow controlled rates of cooling usually in the range of 0.1-0.3°C/min. slowly dehydrate the cell during freezing to prevent intracellular crystallization. Because of this, cryopreservation of oocytes, embryos and other developmental cells using such procedures results in a reduced ability to both establish and maintain pregnancy following transfer. Oocytes are particularly susceptible to cryopreservation damage because of disruption of the metaphase spindle microtubule integrity during cooling.

Alternative prior cryopreservation methods have relied on vitrification with high concentrations of cryoprotectants, which when rapidly cooled result in a glass-like state. However, a disadvantage of this vitrification technique is that the cryoprotectants are very toxic to oocytes, embryos and other delicate developmental cells. Cryoprotectant toxicity can be minimized by increasing the cooling rate, which has been accomplished by plunging oocytes held on electron microscopy grids, or within thinly walled straws (known as open pulled straw) directly into liquid nitrogen. However, both of these procedures are cumbersome and recovery of embryos is problematic.

Therefore a need remains for a method for the vitrification of a biological specimen which is able to maximize the cooling rate of the cells of the specimen; maintain viability of the specimen during vitrification and subsequent thawing; prevent mechanical stress to the specimen; and provide ease of manipulations during cryopreservation and recovery.

## SUMMARY OF THE INVENTION

The present invention relates to a method of vitrification of a biological specimen. According to the method of the present invention, a biological specimen is directly exposed to a freezing material. Upon exposure to the freezing material, the biological specimen undergoes vitrification. The biological specimen which has undergone vitrification may be stored for a period of time, and then thawed at a later date. The thawed biological specimen remains viable. Preferred biological specimens according to the present invention are developmental cells.

The present invention is also drawn to a method of vitrification of a biological specimen, which includes using a transfer instrument to place the biological specimen into a freezing material, such as liquid nitrogen, such that the biological specimen is directly exposed to the freezing material. The biological specimen then undergoes vitrification while held by the transfer instrument, with a loop being a preferred transfer instrument. The transfer instrument and biological specimen are then preferably kept within the freezing material, and transferred into a container which holds a freezing material. The container is preferably a vial. The vial is then sealed containing the freezing material, loop and the vitrified biological specimen, and may be cryopreserved until such time as the biological specimen is required for further use.

Another aspect of the present invention is the treatment of the biological specimen in a cryoprotectant prior to vitrification.

The invention also relates to a method for thawing a biological specimen which has undergone vitrification. The thawing methodology comprises the removal of the biological specimen from the freezing material wherein it has been cryopreserved, and placing the biological specimen in a warmed thaw solution. The thaw solution may be present in any suitable container, and is preferably located within a culture dish or a straw.

A further aspect of the present invention is a method of vitrification of developmental cells, wherein one or more developmental cells are placed directly into a freezing material, such that each developmental cell is directly exposed to the

freezing material thereby undergoing vitrification, wherein the vitrified developmental cells, when thawed, cultured and implanted into suitable host organisms, will result in a fertility rate equal to that of the same developmental cells which had not been vitrified. Preferably, the developmental cells are contained  
5 within a loop when exposed to the freezing material.

The present invention also relates to a method of vitrification of a mammalian blastocyst or mammalian cleavage stage embryo which comprises placing one or more blastocysts or cleavage stage embryos directly into a freezing material, such that each blastocyst or cleavage stage embryo is directly exposed to  
10 the freezing material thereby undergoing vitrification, wherein at least 80 percent, and more preferably, 90 percent, of the vitrified blastocysts or cleavage stage embryos will be viable after being thawed and cultured, preferably in the appropriate base medium. Preferably, the blastocyst or cleavage stage embryo is contained within a loop when exposed to the freezing material.

15 The present invention also relates to a method of vitrification of a horse embryo or pig embryo which comprises placing one or more embryos directly into a freezing material, such that each embryo is directly exposed to the freezing material thereby undergoing vitrification, wherein at least 25 percent, and more preferably, 50 percent, of the vitrified embryos will be viable after being thawed  
20 and cultured, preferably in the appropriate base medium. Preferably, the embryo is contained within a loop when exposed to the freezing material.

The present invention also relates to a kit for the vitrification of a biological specimen. The kit will generally contain instructions describing the vitrification of a biological specimen wherein the specimen is directly exposed to a  
25 freezing material. The kit will also include one or more optional ingredients, including, but not limited to, a transfer instrument, most preferably a loop, a vial which is of the proper size and shape to hold the loop and the vitrified specimen it contains, a base medium, a transfer solution, and a cryoprotectant.

The present invention is also drawn to biological specimens which  
30 have undergone vitrification by the methods of the present invention.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic illustrating a method of vitrification of a biological specimen according to the present invention.

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## DETAILED DESCRIPTION

In the present application, the following terms are used throughout and are defined for the purposes of this application as follows:

**Base Medium:** A solid or liquid preparation made specifically for the growth, manipulation, transport or storage of the biological specimen present therein.

10 **Cryopreservation:** The preservation of a biological specimen at extremely low temperature.

**Developmental Cells:** A reproductive body of an organism that has the capacity to develop into a new individual organism capable of independent existence.

Developmental cells include, but are not limited to, sperm, oocytes, embryos,  
15 morulae, blastocysts, and other early embryonic cells.

**Directly Exposed:** A biological specimen, including blastocysts and embryos, is "directly exposed" to a freezing material if the majority of the surface of the biological specimen, or the medium, solution or material in which the biological specimen resides, is allowed to come into direct contact with the freezing material.

20 **Freezing Material:** Any material, including but not limited to, liquid gases such as liquid nitrogen, liquid propane, liquid helium or ethane slush, which are capable of causing vitrification of a biological material.

**Loop:** An instrument for the manipulation of small biological samples, generally consisting of a rod shaped handle which holds a piece of nylon or metal wire such as  
25 platinum or nickel-steel, etc., formed into a closed loop at the free end.

**Transfer Instrument:** An instrument used to manipulate a biological specimen into a freezing material which is structured in such a fashion that it encircles and/or holds the biological specimen, and/or the medium, solution or material containing the biological specimen, in place during the vitrification process and/or allows ease of manipulation of the biological specimen within the freezing material, and wherein the transfer instrument allows the biological specimen to be directly exposed to the freezing material. The transfer instrument may be any such instrument generally known in the art, including, but not limited to a loop, net with handle, or paddle with handle instrument. The term "transfer instrument" as defined herein does not include either electron microscopy grids or straws (including both sealed straws and open pulled straws).

**Viable:** A biological specimen which is able to live and develop normally for a period of time.

**Vitrification (Vitrify):** A phenomenon wherein a biological specimen is rapidly cooled to very low temperatures such that the water in the specimen forms a glass-like state without undergoing crystallization.

The present invention is directed to a method for the vitrification of biological specimens, based on United States Provisional Patent Application No. 60/104,266, the entire contents of which is hereby incorporated by reference.

According to the method of the present invention, a biological specimen is placed directly into a freezing material such that the biological specimen is directly exposed to the freezing material. Upon exposure to the freezing material, the biological specimen undergoes vitrification. The biological specimen which has undergone vitrification may be stored for a period of time, and then thawed at a later date. The thawed biological specimen remains viable.

The present invention therefore has a number of uses. It may be used for animal husbandry, laboratory research, endangered species preservation, as well as for human assisted reproduction.



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The biological specimen of the present invention can be any sort of viable biological specimen which is a living cell, but is preferably developmental cells, and more preferably mammalian developmental cells. Such cells can include, but are not limited to, sperm, embryos, blastocysts, morulae, and oocytes. Such preferred cells can be from any desired mammalian source, including but not limited to: humans; non-human primates such as monkeys; laboratory mammals such as rats, mice and hamsters; agricultural livestock such as pigs, sheep, cows, goats and horses; and zoologically important and/or endangered animals, etc. The use of other developmental cells from other living creatures are also within the scope of this invention, such as reptiles, amphibians, and insects such as *Drosophila*. Other suitable cells for use with the present invention include both stem cells, including human stem cells, and plant tissue cells. The following Examples describe the use of the present invention with a number of different cell types, including Hamster embryos, which are extremely sensitive to injury and therefore make a good model for any cryopreservation technique. The Examples also show the efficacy of the present invention with bovine oocytes and embryos which are known in the art to be extremely sensitive to chilling injury.

Preferably, the biological specimen is placed on a transfer instrument prior to vitrification. The transfer instrument can be any instrument that allows the biological specimen to be transported into a freezing material, while allowing the biological specimen to be directly exposed to the freezing material, allowing the biological specimen to be cooled very quickly, thus allowing the biological specimen to vitrify rather than form ice crystals within the cell, which would in turn ultimately disrupt cell walls and other vital cellular constituents.

The method of the present invention is in contrast to previous prior art methods wherein the biological specimen was enclosed within a container such as a sealed straw or open pulled straw, rather than being directly exposed to the freezing material.

Additionally, the present methodology differs from previous prior art methods which placed the biological specimen on open plates such as microscopy

grids, which were unable to allow for facile manipulation of the specimen when contained within the freezing material, making handling of the specimen difficult and ultimately resulting in a poor recovery of the vitrified specimen. The present invention therefore allows better handling of the biological specimen during the  
5 vitrification process, and thereby solves the problem of specimen recovery known in prior microscopy grid vitrification methods.

The transfer instrument according to the present invention encircles and/or holds the biological specimen in place during the vitrification process, so that the biological material is not lost during the process. Therefore, the transfer  
10 instrument does not just allow the biological specimen to rest upon it, as with flat sheets or microscopy grids, but may actually help keep the specimen in place, as in the case with a loop via strong adhesion forces which surround the biological specimen, or medium, solution or material containing the specimen. Preferred transfer instruments of the present invention include, but are not limited to, loops,  
15 small nets with an attached handle and small spatulas. The spatulas, nets or loops may be modified in any way known in the art to help retain the biological specimen in place, including the placement of extra polymeric mesh or wire grids within the loop, net or spatula. In a preferred embodiment, the loop has an open loop and is attached via the rod-shaped end directly to the inside of a cap of a vial, the vial  
20 having the appropriate size and shape to allow the vitrified biological specimen and loop to be cryopreserved therein. It has been surprisingly and unexpectedly discovered that the use of a loop in the present vitrification methodology allows fast cooling rates, ease of visualization, facile manipulations and a high success rate of viability when the vitrified specimen is thawed and cultured.

25 In a preferred embodiment, the biological specimen is treated with a small amount of a cryoprotectant prior to vitrification. The methodology of the present invention also allows for a decrease in the time of exposure of the biological specimen to the solution phase of the cryoprotectant used, thus lowering the toxicity of the cryoprotectant to the biological specimen. Cryoprotectants, such as ethylene  
30 glycol, polyethylene glycol, dimethylsulfoxide, glycerol, propane diol, sugars, and

methyle pentane diol, as well as others well known in the art, can be toxic to sensitive cells such as oocytes and embryos when used in large dosages during cryopreservation. The present invention allows for the use of any optional cryoprotectant to be present in solution phase in the presence of the biological specimen for shorter time periods than cryopreservation methods previously described in the art.

By allowing for quick cooling times, reduced time of exposure of solution phase cryoprotectants, and reliable retention and manipulation of the biological specimen, the present invention solves a long standing problem in the art of successful cryopreservation of sensitive biological specimens such as developmental cells. As further described in the Examples, the present invention has shown a success rate of vitrifying blastocysts or cleavage stage embryos such that, when thawed and cultured in the appropriate base medium, the cryopreserved blastocysts or cleavage stage embryos have a viability rate of 80 percent, preferably 90 percent, and preferably greater than 90 percent. Moreover, the present invention allows the vitrification of developmental cells, wherein the vitrified developmental cells, when thawed, cultured and implanted into suitable host organisms, will result in a fertility rate equal to that of developmental cells which are similarly implanted and which have not been cryopreserved. This helps solve a long-term problem in low pregnancy rates resulting from the use of cryopreserved developmental cells.

Additionally, the present methodology allows for the cryopreservation of biological specimens which in the past had resisted efforts of cryopreservation to result in a useful percentage of viable preserved specimens. Notably, pig embryos and horse embryos can now be vitrified according to the present invention, and wherein at least 25, 30, 35, 40, or 45 percent, and more preferably, 50, 55, 60, 65, 70 or 75 percent, of the vitrified embryos will be viable after being thawed and cultured.

According to the present invention, one or more biological specimens are collected by any means well known in the art, and are preferably transferred to a base medium. The base medium may contain one or more optional ingredients,

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such as a cryoprotectant to protect the biological specimen from cold and/or a viscosity increasing compound, to assist in maintaining the material within a transfer instrument, preferably a loop. The viscosity increasing compound can be any such compound known in the art, including, but not limited to, Ficoll, Percoll, 5 hyaluronic acid, albumin, polyvinyl pyrrolidone (PVP), and glycerol.

According to the present invention, the biological specimen is preferably placed on a transfer instrument. The specimen may be placed in a base medium, and the transfer instrument, such as a loop or paddle, used to scoop the biological specimen from the base medium. In a preferred embodiment, the transfer 10 instrument is a loop, and the loop is preferably dipped in base medium to form a thin film of the base material on the loop, and the biological material is deposited via pipette directly into the loop. If developmental cells such as embryos, sperm or oocytes are utilized as the biological specimen, one or more may be placed within each loop.

15 The transfer instrument containing the biological specimen is then quickly placed in a freezing material, such that the biological specimen is directly exposed to the freezing material, allowing vitrification of the biological specimen. Preferably, the time between pipetting the biological specimen onto the transfer instrument and the placement of the biological specimen into the freezing material is 20 45 sec. or less, more preferably 30 sec. or less. The freezing material may be liquid nitrogen, ethane slush, or any other freezing material well known in the art. Preferably, the biological specimen is held within the freezing material during all manipulations subsequent to vitrification, until the specimen is to be thawed.

The vitrified biological specimen is then transferred into a storage 25 container. In a preferred embodiment, the transfer instrument is a loop which is attached to the inside of a vial cap. The vial is filled with the freezing material, and resides in the same reservoir as the freezing material used for vitrification of the biological specimen. After vitrification, the biological specimen, still contained within the loop, may be sealed in the vial without having been removed from the

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freezing material. The sealed vial, which contains the vitrified biological specimen and the loop within the freezing material, can then be cryopreserved indefinitely.

Thereafter, the biological specimen may be thawed, and the viable biological specimen may be further developed. Thawing is accomplished by

5 removing the vial containing the vitrified biological specimen from any storage tank in which it resides, and quickly removing the transfer instrument containing the biological specimen from the vial, and plunging the transfer instrument and specimen into a thaw solution. In a preferred embodiment, the storage vial is placed in a reservoir containing a freezing material, preferably the same freezing material

10 as contained within the vial. While within the freezing material, the vial is opened and the transfer instrument, containing the biological specimen, is removed and quickly plunged into the thaw solution. The thaw solution may be any solution or material that is sufficient to allow the biological specimen to thaw while preserving its viability, including but not limited to, media known in the art that is appropriate

15 as a base medium for the particular biological specimen. After thawing, the biological specimen can be further manipulated in any appropriate manner known for the species and process for which the specimen is being utilized.

A preferred method of the present invention is further illustrated in accordance with FIG. 1. As shown in I, a biological sample in an appropriate base

20 medium is applied directly to a loop or scooped directly into the loop. As shown in I, the loop is attached to a magnetic vial cap. Immediately thereafter, the loop is plunged directly into the freezing material contained within a reservoir, which as illustrated may be a insulated box filled with liquid nitrogen. Alternatively, the freezing material may be placed directly into the vial, and the biological specimen

25 may be vitrified by being directly exposed to the freezing material within the vial itself, thereby eliminating the need for a separate reservoir. While under the liquid nitrogen, the loop is secured into the storage vial, with the vitrified biological specimen remaining within the loop. Multiple vials can be filled by keeping them upright in vial-sized holes within the reservoir, or, alternatively, single vials could

30 be held under the nitrogen with a forceps or other tool. Multiple vials can then be

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cryopreserved indefinitely in any suitable container, such as a standard dewar, as illustrated in III. At any time thereafter, the loop may be removed from the vial while under a freezing material such as liquid nitrogen, as shown in IV, in the exact reversal of the vitrification procedure as described above. It is convenient, but not  
5 necessary, to use a reservoir of freezing material to surround the vial, before thawing, but the freezing material contained within the vial itself should be sufficient to keep the biological specimen cryopreserved during manipulation prior to being thawed. The biological specimen is then plunged directly into a thaw solution. The thaw solution may be contained in any manner which is convenient,  
10 including an open culture dish as shown in V, or in a straw for direct loading into a transfer gun. The biological specimen is instantly diluted into the thaw solution, and floats away from the loop. The biological specimen can then be cultured in any appropriate fashion known in the art.

Vitrification of sensitive biological specimens such as sperm, oocytes  
15 and embryos using the method of the present invention has advantages over conventional cryopreservation procedures in that the present method lacks any insulating layer between the biological specimen and the freezing material. This factor, coupled with the very small volume of less than 1-5  $\mu$ l for the typical biological specimen used, or media, solution or other material containing the  
20 biological specimen used, results in both very rapid and uniform heat exchange during cooling. High rates of cooling prevent chilling injury to sensitive cells such as developmental cells. The extremely rapid cooling rate obtained with the present invention also substantially reduces the exposure time to any optional cryoprotectants used and thereby reduces their cytotoxicity to the specimen.

25 Other major benefits of the methods of the present invention include: an open system enabling ready visualization of the sample during manipulation; rapid freezing of a large number of samples with no need for expensive or complicated equipment; very straightforward labeling and storage; and trivial and instantaneous sample warming and recovery. For applications that require a closed  
30 system such as human clinical applications, the use of standard cryovials enable

them to be closed in standard plastic sheets, or alternatively, the release hole within the cryovial cap may be sealed shut, preventing any possible viral cross-transmission.

The ultimate test of viability of embryos following cryopreservation is the ability to establish and maintain a pregnancy resulting in normal fertile young. The hamster is a good model for this for two reasons. Firstly their sensitivity to the *in vitro* environment makes them a very sensitive model as evidenced by the fact that the inventors of the present invention are unaware of any report in the literature that has successfully produced hamster pups following cryopreservation using any method. Secondly, the hamster has a gestation period of only 16 days and sexual maturity is reached after 3-4 months. The following Examples demonstrate the success of the present methodology for cryopreservation of viable embryos which can then be thawed and used to produce normal young, resulting in a success rate of at least 90%.

Bovine embryos and in particular bovine oocytes are reported to be very sensitive to chilling injury. Furthermore, the high lipid content in the embryo has been linked to the increased sensitivity of bovine embryos to cryopreservation procedures. As shown in the following Examples, vitrification using the method of the present invention with oocytes and cleavage stage embryos of bovines allowed subsequent development to the morula/blastocyst stage in culture, with high percentage rates of successful hatching.

The present invention also relates to a kit for the vitrification of a biological specimen. The kit will generally contain instructions describing the vitrification of a biological specimen wherein the specimen is directly exposed to a freezing material. The kit will also include one or more optional ingredients, including, but not limited to, a transfer instrument, most preferably a loop, a vial which is of the proper size and shape to hold the loop and the vitrified specimen it contains, a base medium, a transfer solution, and a cryoprotectant.

This invention is illustrated further by the following nonlimiting Examples. All of the references listed in the application are hereby incorporated by reference.

5    **Example 1 – Methodologies and Materials For Vitrification of Bovine and Hamster Oocytes and Embryos**

**A. Media**

          The medium used in the following Examples was Hamster Embryo Culture Medium-10 (HECM-10), prepared as described by Lane *et al.*, *Mol. Reprod. Dev.* 50:443-450 (1998). For embryo collection and cryopreservation, a  
10    Hepes-buffered modification of HECM-10 where 20 mM NaHCO<sub>3</sub> was replaced with 20 mM Hepes (pH 7.35) was used. Cryoprotectant solutions were added to the medium immediately prior to use. Media for bovine embryo culture were G1.2 and G2.2, as taught by Gardner *et al.*, *Hum. Reprod.* 13:3434-3440 (1998). All salts,  
15    carbohydrates, amino acids, dimethylsulfoxide (DMSO), ethylene glycol and sucrose were purchased from Sigma Chemical Company (St. Louis, MO). Bovine Serum Albumin was purchased from Bayer Diagnostics.

**B. Hamster Embryo Collection and Culture**

          Hamster embryos were collected from super-ovulated females as  
20    previously described by Lane *et al.*, *Mol. Reprod. Dev.* 50:443-450 (1998). Hamster embryos were cryopreserved at either the pronuclear 1-cell or 2-cell stages. Hamster embryos were cultured in HECM-10 as taught by Lane *et al.*, *Mol. Reprod. Dev.* 50:443-450 (1998). Cell number of resultant blastocysts was assessed by propidium iodide staining following triton treatment.

25    **C. *In Vitro* Maturation/*In Vitro* Fertilization/*In Vitro* Culture (IVM/IVF/IVC) of Bovine Embryos**

          Immature bovine oocytes were isolated from ovaries and matured as described by Krisher *et al.*, *Biol. Reprod.* 60:1345-1352 (1999). Mature oocytes



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were either vitrified and thawed, or not subject to vitrification and thawing when used as controls, and fertilized *in vitro*, by the methods taught by Krisher *et al.*, *Biol. Reprod.* 60:1345-1352 (1999). Following fertilization, putative zygotes were isolated and cultured in sequential media G1.2 and G2.2 for 72 hr. in each medium.

- 5 After a total of 144 hr. development to the morula/blastocyst and blastocyst stages was assessed.

#### D. Vitrification Using Loop

- Loops used for vitrification consisted of a nylon loop (20 $\mu$ m width; 0.5-0.7mm diameter) mounted on a stainless steel pipe held by epoxy to the lid of a cryovial (Hampton Research, Laguna Niguel, CA). Oocytes and embryos were
- 10 vitrified using a 2-step loading with cryoprotectants. Initially oocytes and embryos were placed in cryoprotectant solution I which contained 10% DMSO and 10% ethylene glycol for 1-3 min. before being transferred to solution II, which contained 20% DMSO and 20% ethylene glycol, 10 mg/ml Ficoll (MW 400,000) and 0.65 M
- 15 sucrose for approximately 20 sec. Cells are then transferred to the loop that had previously been dipped into solution II to create a thin-film. For hamster embryos, 10-12 embryos were placed on the loop, and for bovine embryos 3-6 embryos were placed on each loop. The embryos suspended in the nylon loop were then plunged directly into liquid nitrogen. By previously submerging the cryovial under liquid
- 20 nitrogen, the loop containing the embryos was plunged into the cryovial containing liquid nitrogen and sealed under liquid nitrogen in one motion.

- Oocytes and embryos were thawed using a 2-step dilution with sucrose. With the cryovial submerged under liquid nitrogen, the vial was opened and the loop containing cells was removed from the liquid nitrogen, and was then
- 25 inserted directly into a well of the base medium containing 0.25 M sucrose. The oocytes/embryos immediately fell from the loop into the thaw solution. Oocytes were moved from this solution after 2 min. and transferred to base medium containing 0.125 M sucrose for a further 5 min. Subsequently, oocytes/embryos were washed twice in the base medium for 5 min. and were then returned to culture.

### E. Vitrification Using Open Pulled Straw Technique

For comparison purposes, hamster embryos were vitrified using the open-pulled straw (OPS) technique described by Vajta *et al.*, *Cryo-Letters* 18:191-195 (1997). Ten-twelve embryos were exposed to a 2-step loading of  
5 cryoprotectants consisting of ethylene glycol and DMSO at the same concentrations as above. Embryos were pipetted into a 1  $\mu$ l drop of the second cryopreservation solution and then loaded into a pulled straw using capillary action and the straw containing the embryos was plunged directly into liquid nitrogen. For thawing, embryos were expelled from the straw by pressure build-up during warming and  
10 thawed as above.

### F. Embryo Transfer

Hamster morulae/blastocysts were transferred to day 3 (-1 day asynchronous) pseudo-pregnant recipients. Eight embryos were transferred to each uterine horn. On day 14 of pregnancy some animals were euthanized and  
15 implantation and fetal development rates determined. The remaining females were allowed to litter on day 16 of pregnancy and the number of pups was recorded soon after birth.

### G. Statistical Analyses

Differences in development among treatments were assessed using  
20 linear-logistic regression where the distribution was binomial (Glim 4.0, Numerical Algorithms Group, Oxford, UK). Day of experiment was fitted as a factor. Differences in cell numbers were assessed using Analysis of Variance as both Gaussian normality and equal variances were confirmed. Multiple comparisons between treatments were assessed by Bonferroni's procedure for multiple  
25 comparisons.

### Example 2 - Vitrification and Subsequent Development of Hamster Embryos

Hamster 2-cell embryos were vitrified using a loop according to the method of the present invention, and compared with results of control embryos

exposed to cryoprotectant or embryos vitrified using the OPS method, as described in Example 1. Hamster embryos were collected from the oviduct, and allocated to either the control, loop or OPS vitrification. Significantly more embryos developed to the morula/blastocyst and blastocyst stage when vitrified within the loop  
5 compared to those vitrified using OPS, as shown below in Table 1.

Significantly fewer 2-cell embryos were able to continue development to the morula/blastocyst or blastocyst stages in culture following vitrification by either technique compared to control embryos, as shown in Table 1. However, the cell numbers of the blastocysts (an indicator of cleavage rates) resulting from  
10 vitrified 2-cell embryos were statistically equivalent to 2-cell embryos that were not vitrified, as shown in Table 1. Rat 2-cell embryos were also successfully vitrified using the loop and could develop normally after thawing with cleavage rates of 75%, similar to control embryos (n=10).

To further assess the ability to vitrify sensitive embryos, the  
15 experiment was repeated with 1-cell embryos, although the length of time that the 1-cell embryos were exposed to the initial dilution of cryoprotectant was reduced from 2 min. to 1 min. Preliminary studies demonstrated that a 2 min. exposure (without vitrification) of 1-cell embryos to the cryoprotectant solutions severely reduced development. Again embryos were collected from the oviduct and allocated to  
20 either the control, loop or OPS vitrification.

Hamster 1-cell embryos were able to cleave and continue development in culture to the morula/blastocyst stage following vitrification with the loop, as shown in Table 1. Developmental rates after vitrification were significantly better for embryos vitrified using the loop compared to those vitrified using OPS  
25 (Table 1). Hamster oocytes were also able to be successfully vitrified using the loop (n=20) and subsequently fertilized and developed to the morula/blastocyst stage at rates of around 10%, comparable to control non-cryopreserved oocytes.

**Table 1. Development of Hamster Embryos in Culture Following Vitrification**

Stage of Development	Treatment	M/B (%)	B (%)	Blastocyst Cell Number (mean $\pm$ sem)
1-cell	Control	79.5	30.1	18.9 $\pm$ 3.1
	Loop	39.8 <sup>a</sup>	15.5 <sup>a</sup>	11.9 $\pm$ 1.1 <sup>a</sup>
	OPS	22.0 <sup>b</sup>	5.0 <sup>b</sup>	9.2 $\pm$ 1.2 <sup>b</sup>
2-cell	Control	98.5	94.2	24.1 $\pm$ 2.8
	Loop	64.2 <sup>a</sup>	43.1 <sup>a</sup>	19.7 $\pm$ 1.8 <sup>a</sup>
	OPS	50.5 <sup>a</sup>	29.6 <sup>b</sup>	19.6 $\pm$ 1.4 <sup>a</sup>

M/B morula/blastocyst development

B blastocyst development

N = at least 100 embryos cultured per treatment for 1-cell embryos (4 replicates) and at least 400 embryos per treatment for 2-cell embryos (8 replicates)

<sup>a</sup> significantly different from control (P < 0.05)<sup>b</sup> significantly different from control and from loop vitrification (P < 0.05).

10 **Example 3 - Viability of Hamster 1-Cell and 2-Cell Embryos Following Vitrification**

Hamster embryos were vitrified using either the loop method or by OPS. Following warming, embryos were cultured to the morula/blastocyst stage (both vitrified and control embryos) before transfer to pseudo-pregnant recipients.

- 15 There was no difference in the viability of morula/blastocyst stage embryos that had been previously vitrified at the 2-cell stage to implant and develop to a viable fetus compared to control embryos that were not cryopreserved, as shown below in Table 2. However, significantly fewer embryos were able to implant and develop to a viable fetus when vitrified using OPS, as shown in Table 2. Two additional females
- 20 that received morulae/blastocysts which were vitrified at the 2-cell stage using the loop were allowed to litter and 5 normal pups were born. These pups developed into morphologically sound and fertile adults.

Similarly, for hamster 1-cell embryos, implantation and fetal development were not affected by vitrification using the loop, as shown in Table 2.

No embryos vitrified using OPS were transferred due to the low survival rates in culture observed in the previous experiment. Again two females which received morulae/blastocysts vitrified at the 2-cell stage using the loop were allowed to litter and a total of 9 pups were born. One pup was eaten by the mother 6 to 9 days after birth. The remaining pups developed into morphologically sound and fertile adults.

**Table 2. Development of Hamster Embryos *in utero* Following Vitrification**

Stage of Development for Vitrification	n	Method	Implantation N(%)	Fetuses N(%)
1-cell	20	Control	8(40)	6(30)
	17	Loop	7(41)	5(29)
2-cell	40	Control	34(85)	26(65)
	72	Loop	39(54) <sup>a</sup>	36(50)
	112	OPS	48(43) <sup>a</sup>	40(36) <sup>a</sup>

<sup>a</sup> significantly different from control embryos ( $P < 0.05$ )

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**Example 4 - Vitrification of Bovine Oocytes, Cleavage Stage Embryos and Blastocysts**

To determine the ability of the vitrification method of the present invention to successfully vitrify embryos with different cellular properties, bovine oocytes and embryos were vitrified as taught in Example 1, and their survival and subsequent development assessed, and the results shown in Table 3. Oocytes and embryos were allocated to either the control group or to loop vitrification using the methodology of the present invention. *In vitro* produced bovine blastocysts were successfully vitrified using the loop with more than 80% of expanded blastocysts being able to both re-expand and hatch following vitrification, as shown in Table 3. Culture of control blastocysts resulted in 100% hatching after 48 hr. of culture. Furthermore, 75% of completely hatched blastocysts could also be successfully vitrified using loop vitrification. Eight-cell bovine embryos vitrified using the vitrification method of the present invention could be vitrified and warmed with

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subsequent survival rates (assessed by development to the morula/blastocyst and blastocyst stages) equivalent to those obtained for fresh embryos that had not been cryopreserved, as shown in Table 3. Vitrification of embryos at the 4-cell stage resulted in slightly reduced survival rates compared to the fresh embryos, however many were able to complete normal development to the morula/blastocyst stage, as shown in Table 3. Bovine oocytes are extremely sensitive to chilling damage and few reports have demonstrated any success following cryopreservation. *In vitro* matured bovine MII oocytes were successfully vitrified using the loop. Vitrified and warmed oocytes were subsequently fertilized and of these 33 % continued development to the morula/blastocyst stage (n=42).

**Table 3. Development of Bovine Embryos in Culture Following Vitrification**

Stage of Development	Treatment	8-Cell at 72 Hr. (%)	M/B at 144 Hr. (%)	B at 144 Hr. (%)	HB at 168 Hr. (%)
4-cell	Control	0	46	15	n/d
	Loop	4	24	16	n/d
8-cell	Control	n/a	59	50	n/d
	Loop	n/a	52	41	n/d
Blastocyst	Control	n/a	n/a	n/a	100
	Loop	n/a	n/a	n/a	80.5

M/B, morula/blastocyst development

B blastocyst development

15 HB hatched blastocyst development

n/a not applicable

n/d not determined

20 **Example 5 - Methodologies and Materials For Vitrification of Human and Mouse Blastocysts**

**A. Culture Media**

Media for embryo culture was G1.2 and G2.2 (IVF Sciences Scandinavian, Gothenburg, Sweden). Media for embryo collection was a HEPES-

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modification of G1.2 (H-G1.2) and the base medium for cryopreservation and thawing was a HEPES- buffered modification of G2.2 without amino acids and vitamins (H-G2.2). In both cases the media were modified by replacing 20 mM  $\text{NaHCO}_3$  with 20 mM HEPES and adjusted to pH 7.35.

## 5 B. Mice

Embryos were collected from 4-6 week old F1 (C57BL6xCB<sub>a</sub>) females. Females were stimulated with 5 iu of pregnant mare's gonadotrophin (Sigma Chemical Co., St. Louis, MO) and 48 hr. later with 5 iu of human chorionic gonadotrophin (hCG; Sigma Chemical Co.). Following the hCG injection females  
10 were placed with males of the same strain and the following morning the presence of a vaginal plug indicated that mating had taken place. Zygotes were collected at 22 hr. post-hCG and denuded from surrounding cumulus by incubation in H-G1.2 with 0.5 mg/ml hyaluronidase for less than 1 min. Zygotes were washed twice in H-G1.2 and placed in culture.

## 15 C. Mouse Embryo Culture

Mouse zygotes were cultured in groups of 10 in 20  $\mu\text{l}$  drops of medium G1.2 at 37°C in an humidified atmosphere of 5%  $\text{CO}_2$  in air. After 48 hr. of culture, 8-cell embryos were washed 3 times in medium G2.2 and cultured for a further 48 hr. in 20  $\mu\text{l}$  drops of medium G2.2. Blastocyst development was assessed  
20 after 96 hr. of culture.

## D. Human Embryo Culture

The culture system for blastocyst growth was done according to Gardner *et al.*, *Hum. Reprod.* 13:3434-40 (1998). Following oocyte retrieval, cumulus enclosed oocytes were incubated in Ham's F-10 supplemented with fetal  
25 cord serum (FCS) for insemination. Semen was prepared with a 50-70-95 discontinuous gradient or mini-gradient method (Pure Sperm, Nidacon, Gothenburg), depending on the initial semen parameters. The resulting pellet was washed in Ham's F-10. For normal insemination, to each oocyte, 50-100,000

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sperm/mL were added. If intracytoplasmic injection (ICSI) was performed, oocytes were denuded using hyaluronidase and drawn pipettes. Each mature oocyte was placed in a 6  $\mu$ l droplet of phosphate buffered saline supplemented with 15% FCS. The partner's sperm was placed in a 6  $\mu$ l droplet of PVP (IVF Sciences Scandinavian). All droplets were overlaid with Ovoil (IVF Sciences Scandinavian). ICSI was performed on a Nikon inverted microscope with Narishige micromanipulators. Injected oocytes were then rinsed and placed in tubes of G 1.2 until fertilization was assessed. Fertilization was assessed 15-18 hr. post insemination or ICSI. Cumulus and corona cells were removed by dissection with 27-gauge disposable needles in an organ culture dish. Resulting 2 pronuclear embryos were washed well and subsequently cultured in groups of 2-3 in G1.2 medium in 1-mL Falcon culture tubes in 5% CO<sub>2</sub> in air.

After 48 hr. of culture, embryos were rinsed 3 times and cultured for a further 72 hr. Blastocysts on day 6 that were not considered of good enough quality to cryopreserve by previous methods known in the art, i.e. not fully expanded or with poor inner cell mass development, were donated for vitrification by the method of the present invention.

#### E. Vitrification using a Loop

Loops used for vitrification consisted of a nylon loop (20 $\mu$ m width; 0.5-0.7mm diameter) mounted on a stainless steel pipe inserted into the lid of a cryovial. The loops were purchased mounted (Hampton Research, Laguna Niguel, CA) and then epoxied into vials. A metal insert on the lid enables the use of a handle with a small magnet for manipulation of the loop if desired.

Blastocysts were vitrified using a 2-step loading with cryoprotectants. Initially blastocysts were placed in cryoprotectant solution I which contained 10% DMSO and 10% ethylene glycol for 2 min. before being transferred to solution II, which contained 20% DMSO and 20% ethylene glycol, 10 mg/ml Ficoll (MW 400,000) and 0.65 M sucrose for around 20 sec. These concentrations of cryoprotectants and length of exposure have previously been demonstrated to be optimal for the vitrification of both rodent and domestic animal embryos using the



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loop procedure. While blastocysts are in cryoprotectant solution I, the loop is dipped into cryoprotectant solution II to create a thin film on the loop. The blastocysts were then transferred from solution II onto the film of cryoprotectant on the loop. The loop containing the blastocyst was then plunged into the cryovial  
5 which is submerged and filled with liquid nitrogen. By previously submerging the cryovial under liquid nitrogen, the loop containing the blastocysts could be plunged into the cryovial containing liquid nitrogen and sealed under liquid nitrogen in one motion. The vials were stored in standard canes.

Blastocysts were thawed using a 2-step dilution with sucrose. With  
10 the cryovial submerged under liquid nitrogen the vial was opened and the loop containing blastocysts removed from the liquid nitrogen and placed directly into a well of the base medium containing 0.25 M sucrose. The blastocysts immediately fell from the loop into the thaw solution. Blastocysts were moved from this solution after 2 min. and transferred to base medium containing 0.125 M sucrose for a  
15 further 3 min. Subsequently, blastocysts were washed twice in the base medium for 5 min. and were then returned to culture.

Following vitrification, mouse and human blastocysts were cultured in medium G2.2 for 6 hr. to assess re-expansion before assessment of blastocyst outgrowth. A 6 hr. incubation was chosen as this is the normal time period used for  
20 the assessment of thawed blastocysts prior to transfer.

#### **F. Assessment of Blastocyst Outgrowth**

Both mouse and human blastocysts were assessed for outgrowth as a marker of subsequent viability. Blastocysts were transferred to medium G2.2 supplemented with 10% fetal cord serum to assess blastocyst attachment and  
25 outgrowth. Blastocysts were cultured in 4 well plates (Nunclon, Denmark) previously coated with 0.1 % gelatin in 500  $\mu$ l drops at 37°C in 5 % CO<sub>2</sub> in air for 48 hr. Blastocyst hatching and attachment were assessed after 24 hr. and outgrowth assessed after a further 24 hr. of culture. Outgrowth of inner cell mass (ICM) and trophectoderm was given a score between 0 and 3 based on the amount of

outgrowth, where 0 was no growth and 3 was extensive growth as described by Spindle and Pederson, *J. Exp. Zool.*, 186:305-318 (1972).

#### G. Assessment of Blastocyst Viability in Mice

Viability of mouse blastocysts following vitrification was assessed by transfer to pseudo-pregnant recipients. Following warming, blastocysts were cultured for 6 hr. in medium G2.2 prior to transfer. All blastocysts that re-expanded after the 6 hr. period were pooled and blastocysts for transfer randomly selected. Six blastocysts were transferred to each uterine horn. On day 15 of pregnancy, implantation, fetal development and fetal weights were assessed. Non-cryopreserved blastocysts served as the control.

#### H. Statistical Analyses

Differences in hatching, attachment and viability following vitrification were assessed by Chi-square analysis with Yates Correction. Data for outgrowth of both the ICM and trophectoderm were initially subjected to a Kolmogorov-Smirnov test to determine the normality of the data. An F-test was then used to assess that the two groups of data had equal variances. Once the normality and equal variances were established differences in outgrowth were assessed by Student's t-test.

#### 20 Example 6 - Vitrification of Mouse Blastocysts

A total of 160 mouse blastocysts were vitrified using a loop according to the present invention. Following vitrification, 100% of these blastocysts were able to re-expand in culture. There was no difference in the ability of vitrified blastocysts to hatch and attach in culture compared to control embryos, as shown in Table 4. Similarly, there was no difference in the ability of either the ICM or trophectoderm to outgrow in culture between the control and vitrified blastocysts, as demonstrated in Table 4.

Following vitrification and thawing, 60 blastocysts were transferred to pseudo-pregnant recipients and their viability compared to sibling control

blastocysts that were not cryopreserved. There was no difference in the ability of vitrified blastocysts to implant and develop to a fetus compared to control blastocysts. Resultant fetal weights were also similar for blastocysts that were vitrified ( $0.245 \pm 0.021$  g) compared to control blastocysts ( $0.250 \pm 0.017$  g). All  
 5 fetuses resulting from both vitrified and control blastocysts were morphologically normal. Additionally a recipient female that received 8 vitrified blastocysts (4 per uterine horn) was allowed to litter. Three morphologically normal pups were born.

10 **Table 4. Effect of Loop Vitrification of Mouse Blastocysts on Re-Expansion and Outgrowth**

Treatment	Study group	
	Control	Vitrified blastocysts <sup>1</sup>
Re-expansion (%)	-	100
Hatching (%)	87.5	95.5
Attachment (%)	78.1	85.9
ICM outgrowth (means $\pm$ SEM)	$2.21 \pm 0.10$	$2.17 \pm 0.09$
trophectoderm outgrowth (means $\pm$ SEM)	$2.00 \pm 0.09$	$2.14 \pm 0.09$

<sup>1</sup> there was no difference between control and vitrified blastocysts for any parameter measured.

n  $\geq$  100 for both control and vitrified blastocysts

15

#### **Example 7 – Human Blastocyst Vitrification**

Eighteen human blastocysts between minimally to semi-expanded were vitrified using a loop according to the methodology of the present invention. Of these 11 (83.3 %) re-expanded in culture. Ability to hatch in culture and  
 20 outgrowth of the ICM and trophectoderm were similar for blastocysts that were vitrified and control blastocysts that were not cryopreserved, as shown in Table 5.

**Table 5. Effect of Loop Vitrification of Human Blastocysts on Re-Expansion and Outgrowth**

Treatment	Study group	
	Control n = 12	Vitrified blastocysts <sup>1</sup> n = 18
Re-expansion (%)	-	83.3
Hatching (%)	63.6	73.3
Attachment (%)	36.0	60.0
ICM outgrowth (means $\pm$ SEM)	2.0 $\pm$ 0.2	1.7 $\pm$ 0.2
trophectoderm outgrowth (means $\pm$ SEM)	1.7 $\pm$ 0.2	2.0 $\pm$ 0.2

<sup>1</sup> there was no difference between control and vitrified blastocysts for any parameter measured.

5

**Example 8 – Vitrification of 8 Day Bovine Blastocyst**

The following solutions were used in this Example:

**Freeze solutions:**

- 10    Solution 1: Base medium (as taught in Example 1) containing 10% ethylene glycol and 10% DMSO
- Solution 2. Base medium containing 0.65 M sucrose and 20% ethylene glycol and 20% DMSO and 10 mg/ml Ficoll

**Thaw solutions:**

- 15    Solution 1: Base medium with 0.25 M sucrose
- Solution 2: Base medium with 0.125M sucrose
- Solution 3: Base medium
- Solution 4: Base medium

- 20                    Bovine blastocysts were produced according to the methods described in Example 1. The blastocysts were all expanded to some degree prior to

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vitrication. The blastocysts were pipetted using standard equipment onto a loop that had been dipped into Freeze Solution 2. Each loop contained a single blastocyst. The loops containing the blastocysts was then treated for 2 min. in Freeze Solution 1, followed by 30 sec. of treatment in Freeze Solution 2 which  
5 included a viscosity solution of Ficoll and then immediately plunged directly into liquid nitrogen and vitrified. The blastocysts were kept frozen in liquid nitrogen for 30-90 min. and then thawed.

To thaw the blastocysts, the blastocysts and the loop in which they were vitrified were placed for five min. each in Thaw Solutions 1-4 sequentially.

10 Of the 13 blastocysts that were vitrified and thawed according to the present invention, 9 of the blastocysts hatched after being cultured after 48 hr.

A second set of 4 blastocysts were vitrified, thawed and cultured as described above, and all 4 blastocysts successfully hatched after being cultured for 48 hr.

15 A third set of 8-day bovine blastocysts were produced by the methods described in Example 1. A total of 12 blastocysts were used, and 2 expanded blastocysts were placed in each loop. The blastocysts were subject to vitrication, thawing and culturing as described, except the thawing process was conducted according to the following regime. Two min. in Solution 1; 5 min. in Solution 2;  
20 5 min. in Solution 3 and 5 min. in Solution 4. After being cultured for 48 hr., 11 of the blastocysts had hatched.

#### **Example 9 - Vitrification of Nine Day Bovine Blastocysts**

Bovine blastocysts were produced according to methods described in  
25 Example 1 and vitrified as described in Example 8. The blastocysts were then thawed and cultured as described in Example 8. After 48 hr., 80% of the blastocysts had hatched.

**Example 10 – Vitrification of Seven Day Bovine Blastocysts**

Seven day bovine blastocysts were produced as described in Example 1 and cultured using G1.2/G2.2 as described in Example 1. A total of 20 blastocysts were vitrified and thawed according to the method described in Example 8 and frozen for 2 hr. with 2 to 3 blastocysts per loop. The blastocysts were then thawed and cultured as described in Example 8. After the thawed blastocysts were cultured for 48 hr., 15 of the 20 blastocysts had hatched and 2 had re-expanded.

A second experiment was conducted with seven day blastocysts according to the procedures described above, with 1 to 2 blastocysts per loop. After 48 hr. of culture, 29 of the 33 vitrified blastocysts had hatched.

**Example 11 – Vitrification of Bovine Oocytes**

In this Example, the vitrification of bovine oocytes using methodology of the present invention was compared to oocytes which were vitrified using the known open pulled straw (OPS) methodology and also to control oocytes which had not been frozen. The oocytes which were to be vitrified were treated for 35 sec. in Solution 1 and for 30 sec. in either Solution 2 alone or Solution 2 plus a viscosity solution, using the Solutions described in Example 8. The oocytes were then frozen either 1-3 per straw or 1 to 3 per loop by plunging them into liquid nitrogen. After vitrification, all oocytes were thawed according to the following regime: 1 min. in Solution 1; 5 min. in Solution 2; 5 min. in Solution 3; and 5 min. in Solution 4, using the Solutions of Example 8. The bovine oocytes were then returned to maturation media for 2 hr. and then fertilized in the normal fashion. After one day, the oocytes were moved to medium G1.2 as described in Example 1. After 4 days, the cleaved embryos were moved to fresh medium G1.2, as described in Example 1. The control oocytes showed 37.5% cleavage, the oocytes which had undergone vitrification according to the current methodology showed 19% cleavage, and those which were vitrified using the OPS methodology showed 14% cleavage. The oocytes were then moved to fresh G2.2 medium and cultured for an additional 4 days. At the end of eight days, only 2 of the 24 original oocyte denuded controls

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had reached the morula stage of development. In comparison, 3 of the 16 oocytes which had been vitrified according to the present invention had reached the morula stage of development. In comparison, of the 28 oocytes which had been subjected to vitrification using the OPS methodology, none of them had reached the morula stage of development, with only 4 such oocytes surviving, with the most successful single example of the OPS method having progressed to the 16-32 cell stage.

**Example 12 - Methodologies and Materials For the Vitrification of Mouse and Human Oocytes**

10 **A. Media**

Media for embryo culture was G1.2 and G2.2 supplemented with 5 mg/ml human serum albumin (Gardner *et al.*, *Hum. Reprod.* 13:3434-40 (1998)). Media for embryo collection and vitrification was a HEPES- buffered modification of G1.2 without EDTA, modified by replacing 20 mM NaHCO<sub>3</sub> with 20 mM HEPES and adjusted to pH 7.35.

**B. Mice**

Oocytes were collected from 4-5 week old F1 (C57BL6xCB<sub>a</sub>) females. Females were stimulated with 5 iu of pregnant mare's gonadotrophin (Sigma Chemical Co., St. Louis, MO) and 53 hr. later with 5 iu of human chorionic gonadotrophin (hCG; Sigma Chemical Co.). Oocytes were collected at 14 hr. post-hCG and denuded from surrounding cumulus by incubation in H-G1.2 with 0.5 mg/ml hyaluronidase for less than 1 min. Oocytes were washed twice in H-G1.2 and either inseminated or cryopreserved.

**C. Human Oocyte Collection**

25 Patients were stimulated to produce multiple oocytes according to Gardner *et al.*, *Hum. Reprod.* 13:3434-40 (1998). Oocytes were flushed from the follicles and placed into culture at G1.2 for 4 hr. Oocytes were denuded from surrounding cumulus by incubation in G1.2 with hyaluronidase. Immature oocytes

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were then placed into culture in G2.2 with fetal cord serum for 24 hr. Mature MII oocytes were then either allocated to control or were vitrified using the loop.

#### **D. Loop Vitrification of mouse and human oocytes**

Oocytes were vitrified using a 2-step loading with cryoprotectants.

- 5 Initially oocytes were placed in cryoprotectant solution I which contained 10% DMSO and 10% ethylene glycol for 1 min before being transferred to solution II, which contained 20% DMSO and 20% ethylene glycol, 10 mg/ml Ficoll (MW 400,000) and 0.65 M sucrose for around 20 sec. Oocytes were then transferred to the loop that had previously been dipped into solution II to create a thin-film, and  
10 plunged directly into liquid nitrogen. By previously submerging the cryovial under liquid nitrogen, the loop containing the oocytes could be plunged into the cryovial containing liquid nitrogen and sealed under liquid nitrogen in one motion. The vials were stored in standard canes.

- Oocytes were thawed using a 2-step dilution with sucrose. With the  
15 cryovial submerged under liquid nitrogen the vial was opened and the loop containing cells removed from the liquid nitrogen and placed directly into a well of the base medium of H-G1.2 containing 0.25 M sucrose. The oocytes immediately fall from the loop into the thaw solution. Oocytes were moved from this solution after 2 min and transferred to base medium H-G1.2 containing 0.125 M sucrose for  
20 a further 3 min. Subsequently, oocytes were washed twice in H-G1.2 for 5 min. and were then returned to culture.

#### **E. *In Vitro*-Fertilization and Embryo Culture of Mouse oocytes**

- Spermatozoa were aspirated from the epididymis of 12-16 week old F1 (C57BL6xCB<sub>a</sub>) male mice into medium FG1 (as described by Gardner and Lane, 1997, *Hum. Reprod. Update* 3:367-382) supplemented with 1 mg/ml glutathione and  
25 5 mg/ml HSA (Scandinavian IVF Sciences, Gothenburg, Sweden). Spermatozoa were capacitated for 1.5 hr. before insemination. Before oocytes were inseminated, a small hole (5 $\mu$ M) was made in the zona of the oocytes using a Fertilase 670 nm laser aiming beam and a collimated 1.48  $\mu$ M laser beam (MTM Medical



Technologies, Montreux, Switzerland). Oocytes were placed in 100 $\mu$ l drops of FG1 and co-incubated with approximately  $1 \times 10^4$  sperm for 4 hr. Oocytes were washed twice and cultured in 20  $\mu$ l drops of G1.2 at 37°C in 6% CO<sub>2</sub>, 5% O<sub>2</sub> and 89% N<sub>2</sub>. Fertilization was assessed by the presence of 2-cell embryos the following morning.

- 5 All 2-cells were moved to fresh drops of G1.2. After 48 hr. culture embryos were washed well in G2.2 and cultured for a further 48 hr. in medium G2.2 to the blastocyst stage.

#### F. Viability Assessment of Human Oocytes

- Viability of human oocytes was assessed by dye-exclusion. Oocytes  
10 were placed in H-G1.2 containing 25 $\mu$ g/ml of propidium iodide for 10 min., then washed in H-G1.2 for 5 min. Oocytes that had not survived the vitrification procedure had positive staining of nuclei material, while surviving oocytes demonstrate no staining.

#### G. Statistical Analysis

- 15 Differences in development among treatments were assessed using linear-logistic regression where the distribution was binomial (Glim 4.0, Numerical Algorithms Group, Oxford, UK). Day of experiment was fitted as a factor. Differences in cell numbers were assessed using Analysis of Variance as both Gaussian normality and equal variances were confirmed. Multiple comparisons  
20 between treatments were assessed by Bonferroni's procedure for multiple comparisons.

#### Example 13 - Development in Culture of Mouse Oocytes Following Cryopreservation

- 25 Oocytes vitrified using the method of the present invention had significantly higher rates of survival compared to oocytes cryopreserved using the slow-freezing procedure, as shown in Table 6. Similarly, fertilization rates were significantly higher in oocytes that were vitrified using the method of the present invention compared to the slow-freezing procedure as shown in Table 6. Oocytes

5 vitrified using the present method had equivalent fertilization rates to control fresh oocytes which were inseminated, as shown in Table 6. Rates of fertilization were significantly lower in oocytes cryopreserved using the slow-freezing procedure as shown in Table 6. Control embryos that were not frozen developed to the blastocyst stage at rates of 70.0% from total oocyte or 95.4% from 2-cell embryos. There was no difference in blastocyst development rates from oocytes that were vitrified using the loop compared to the control oocytes as shown in Table 6. In contrast, rates of blastocyst development from total oocytes or from 2-cell embryos was significantly reduced in oocytes that were cryopreserved by slow-freezing as shown in Table 6.

10

**Table 6. Effect of Cryopreservation of Mouse Oocytes on Mouse Fertilization and Embryo Development**

Treatment	Survival (%)	Fertilization (%)	Blastocyst/Total (%)	Blastocyst/2-Cell (%)
Control	100	73.4	70.0	95.4
Loop	99.2	69.8	67.4	96.5
Slow-freeze	80.9*	39.5**	25.7**	65.1**

N= at least 300 embryos per treatment

\* significantly different from all other treatments ( $P < 0.05$ )

15 \*\* significantly different from all other treatments ( $P < 0.01$ )

**Example 14 - Subsequent viability of mouse oocytes following cryopreservation**

20 Blastocysts derived from either fresh or cryopreserved oocytes (either by vitrification with the present methodology or slow freezing) were transferred to pseudo-pregnant recipients and implantation and fetal development assessed and the results given in Table 7. Blastocysts that were derived from oocytes that were vitrified using the method of the present invention had similar implantation rates to fresh oocytes, however fetal development was slightly lower. In contrast, oocytes that were frozen using the slow-freeze procedure had significantly reduced implantation and fetal development rates compared to either control oocytes or oocytes vitrified using the method of the present invention. Additionally, 2 females

which each had 8 blastocysts transferred that resulted from oocytes that were vitrified were allowed to litter. Eleven pups were born from these females (7 and 4) and all developed into morphologically normal and fertile adult mice. Of the eleven pups born, 8 were females and 3 males.

5

**Table 7. Effect of Cryopreservation on Mouse Viability Following Transfer**

Treatment	Implantation (%)	Fetal Development (%)	Fetal Development/Implantation (%)
Control	86 <sup>a</sup>	68.0 <sup>a</sup>	79.1 <sup>a</sup>
Loop	88.0 <sup>a</sup>	56.5 <sup>b</sup>	64.2 <sup>b</sup>
Slow-freeze	52.4 <sup>b</sup>	26.2 <sup>c</sup>	50.0 <sup>b</sup>

N= at least 50 blastocysts transferred per treatment group  
a-c different letters are significantly different (P < 0.05)

10

**Example 15 - Survival Rates of Human Oocytes Following Cryopreservation**

Viability of human oocytes was assessed following vitrification by the present methodology. High rates of survival were observed, as shown in Table 8.

15 **Table 8. Survival Rates of Human Oocytes Following Cryopreservation**

Treatment	Number of Embryos	Survival (%)
Fresh oocytes	12	100
Vitrified oocytes	21	81.9

**Example 16 - Vitrification Of Mouse Cleavage Stage Embryos**

Embryos were collected according to the methods described in

20 **Example 1.** One-cell embryos were vitrified using the loop immediately following collection while 2-cell embryos were obtained following 24 hr. culture according to the methods described in Example 1.

One-cell and 2-cell embryos were vitrified using the loop according to the methods described in Example 12. There was no difference in the ability of mouse 1-cell or 2-cell embryos to develop to the blastocyst stage in culture compared to fresh embryos that were not cryopreserved. The results are shown below in Table 9.

**Table 9. Development of Mouse 1-cell and 2-cell Embryos Following Vitrification According to the Present Invention**

Treatment <sup>#</sup>	Number of Embryos	Blastocyst (%)
Fresh 1-cells	20	95.0
Vitrified 1-cells	20	90.0
Fresh 2-cells	30	96.7
Vitrified 2-cells	30	93.3

<sup>#</sup>There was no difference in the ability of loop vitrified 1-cell or 2-cell embryos to develop in culture compared to fresh embryos.

**Example 17 - Vitrification Of Mouse Spermatozoa Using The Present Invention**

Mature mouse spermatozoa were collected according to the methods described in Example 12.

A 1 $\mu$ l drop of cryoprotectant solution I which contained 10% DMSO and 10% ethylene glycol was placed on the lid of a petri dish. A 1 $\mu$ l of sperm solution was added to the drop of solution I. After 20 sec, 1 $\mu$ l of solution II, which contained 20% DMSO and 20% ethylene glycol, 10 mg/ml Ficoll (MW 400,000) and 0.65 M sucrose was added and the whole drop placed on a loop which was plunged into liquid nitrogen. For thawing, the loop was placed into a 20 $\mu$ l of base medium containing 0.25M sucrose for 30 sec., when 20 $\mu$ l of base medium was added to the drop for a further 1 min, finally 2 ml of base medium was added. Following this procedure, viable sperm could be obtained as determined by motility.

It is understood that the invention is not confined to the particular embodiments set forth herein as illustrative, but embraces all such modified forms thereof as come within the scope of the following claims.

## CLAIMS

What is claimed is:

- 1                   1.     A method of vitrification of a biological specimen,  
2     comprising:  
3                   (a)     placing the biological specimen on a transfer instrument;  
4                   (b)     placing the transfer instrument and the biological specimen  
5     directly into a freezing material, wherein the biological specimen is directly exposed  
6     to the freezing material thereby undergoing vitrification, and further wherein the  
7     biological specimen will be viable after the biological specimen is thawed.
- 1                   2.     The method of claim 1, wherein the biological specimen is  
2     selected from the group consisting of an embryo, a sperm, an oocyte, a blastocyst  
3     and a morula.
- 1                   3.     The method of claim 1, wherein the transfer instrument is  
2     selected from the group consisting of a loop, a net and a paddle.
- 1                   4.     The method of claim 1, wherein (a) further comprises:  
2                   (i)     treating the biological specimen with a cryoprotectant  
3     prior to vitrification.
- 1                   5.     The method of claim 1, further comprising:  
2                   (c)     thawing the biological specimen which has undergone  
3     vitrification.
- 1                   6.     The method of claim 1, wherein (b) further comprises:  
2                   (i)     transferring the biological specimen which has  
3     undergone vitrification into a storage container, the storage container  
4     containing a freezing material; and

5 (ii) storing the storage container containing the biological  
6 specimen which has undergone vitrification until the biological specimen is  
7 ready to be thawed.

1 7. The method of claim 5, wherein (c) further comprises:

2 (i) removing the biological specimen from the freezing  
3 material; and

4 (ii) placing the biological specimen in a thaw solution.

1 8. The method of claim 7, wherein the thaw solution is contained  
2 within a culture dish.

1 9. The method of claim 7, wherein the thaw solution is contained  
2 within a straw.

1 10. The method of claim 1, wherein (a) further comprises:

2 (i) placing the biological specimen in a base medium,  
3 wherein the biological specimen is selected from the group consisting of an  
4 embryo, an oocyte, a sperm, a blastocyst and a morula, wherein the transfer  
5 instrument is selected from the group consisting of a loop, a net and a  
6 paddle; and further wherein (b) further comprises:

7 (i) placing the transfer instrument containing the  
8 biological specimen into the freezing material, wherein the freezing material  
9 is located within a container, such that the biological specimen undergoes  
10 vitrification and;

11 (ii) sealing the container which contains the freezing  
12 material, the transfer instrument, and the biological specimen.

1 11. The method of claim 10, wherein the base medium comprises  
2 one or more ingredients selected from the group consisting of a cryoprotectant and a  
3 viscosity increasing compound.

1 12. The method of claim 10, further comprising:

2 (c) thawing the biological specimen which has undergone  
3 vitrification.

1 13. The method of claim 12, wherein (c) further comprises:

2 (i) removing the biological specimen from the freezing  
3 material; and

4 (ii) placing the biological specimen in a thaw solution.

1 14. The method of claim 12, wherein the thaw solution is  
2 contained within a culture dish.

1 15. The method of claim 12, wherein the thaw solution is  
2 contained within a straw.

1 16. A biological specimen which has undergone vitrification  
2 produced by the method of claim 1.

1 17. A biological specimen which has undergone vitrification  
2 produced by the method of claim 11.

1 18. A method of vitrification of a biological specimen,  
2 comprising:

3 (a) placing the biological specimen in a base medium, wherein the  
4 biological specimen is selected from the group consisting of an embryo, an oocyte, a  
5 sperm, a blastocyst and a morula;

6 (b) using a transfer instrument to move the biological specimen  
7 into a freezing material located within a container, such that the biological specimen  
8 is directly exposed to the freezing material and undergoes vitrification;

9 (c) sealing the container which contains the freezing material, the  
10 transfer instrument, and the biological specimen;

11 (d) subjecting the sealed container to storage;

12 (e) removing the biological specimen from the sealed container;

13 and



- 14 (f) placing the biological specimen in a thaw solution.
- 1 19. The method of claim 18, wherein the transfer instrument is a  
2 loop.
- 1 20. A method of vitrification of developmental cells, comprising:  
2 (a) placing one or more developmental cells directly into a  
3 freezing material, such that each developmental cell is directly exposed to the  
4 freezing material thereby undergoing vitrification, wherein the vitrified  
5 developmental cells, when thawed, cultured and implanted into suitable host  
6 organisms, will result in a fertility rate equal to that of developmental cells which  
7 had not undergone vitrification.
- 1 21. The method of claim 20, wherein the developmental cells are  
2 selected from the group consisting of embryos, sperm, oocytes, morulae and  
3 blastocysts.
- 1 22. The method of claim 21, wherein a loop is utilized to  
2 transport the developmental cells into the freezing material.
- 1 23. The method of claim 21, wherein (a) further comprises:  
2 (i) treating the biological specimen with a cryoprotectant  
3 prior to vitrification.
- 1 24. A method of vitrification of a mammalian blastocyst or  
2 mammalian cleavage stage embryo comprising:  
3 (a) placing one or more blastocysts or cleavage stage embryos  
4 directly into a freezing material, such that each blastocyst or cleavage stage embryo  
5 is directly exposed to the freezing material thereby undergoing vitrification, wherein  
6 at least 80 percent of the vitrified blastocysts or cleavage stage embryos will be  
7 viable after being thawed and cultured.

1                   25.    The method of claim 24, wherein at least 90 percent of the  
2    vitrified blastocysts or cleavage stage embryos will be viable after being thawed and  
3    cultured.

1                   26.    The method of claim 24, wherein a loop is utilized to  
2    transport the blastocyst or cleavage stage embryo into the freezing material.

1                   27.    The method of claim 24, wherein (a) further comprises:  
2                   (i)     treating the blastocyst or cleavage stage embryo with a  
3                   cryoprotectant prior to vitrification.

1                   28.    The method of claim 24, wherein the mammal is selected from  
2    the group consisting of humans, rodents and bovines.

1                   29.    A method of vitrification of a horse embryo or pig embryo  
2    comprising:  
3                   (a)     placing one or more embryos directly into a freezing material,  
4    such that each embryo is directly exposed to the freezing material thereby  
5    undergoing vitrification, wherein at least 25 percent of the vitrified embryos will be  
6    viable after being thawed and cultured.

1                   30.    The method of claim 29, wherein at least 50 percent of the  
2    vitrified embryos will be viable after being thawed and cultured.

1                   31.    The method of claim 29, wherein a loop is utilized to  
2    transport the embryo into the freezing material.

1                   32.    The method of claim 29, wherein (a) further comprises:  
2    treating the embryo with a cryoprotectant prior to vitrification.

1                   33.    A kit for the vitrification of a biological specimen,  
2    comprising:  
3                   (a)     a base medium;

-41-

- 4 (b) instructions for vitrifying a biological specimen wherein the  
5 specimen is directly exposed to a freezing material;  
6 (c) a loop; and  
7 (d) a vial, wherein the vial is of an appropriate size and shape for  
8 storing the loop containing a vitrified biological specimen.
- 1 34. The kit of claim 33, further comprising:  
2 (e) a cryoprotectant.

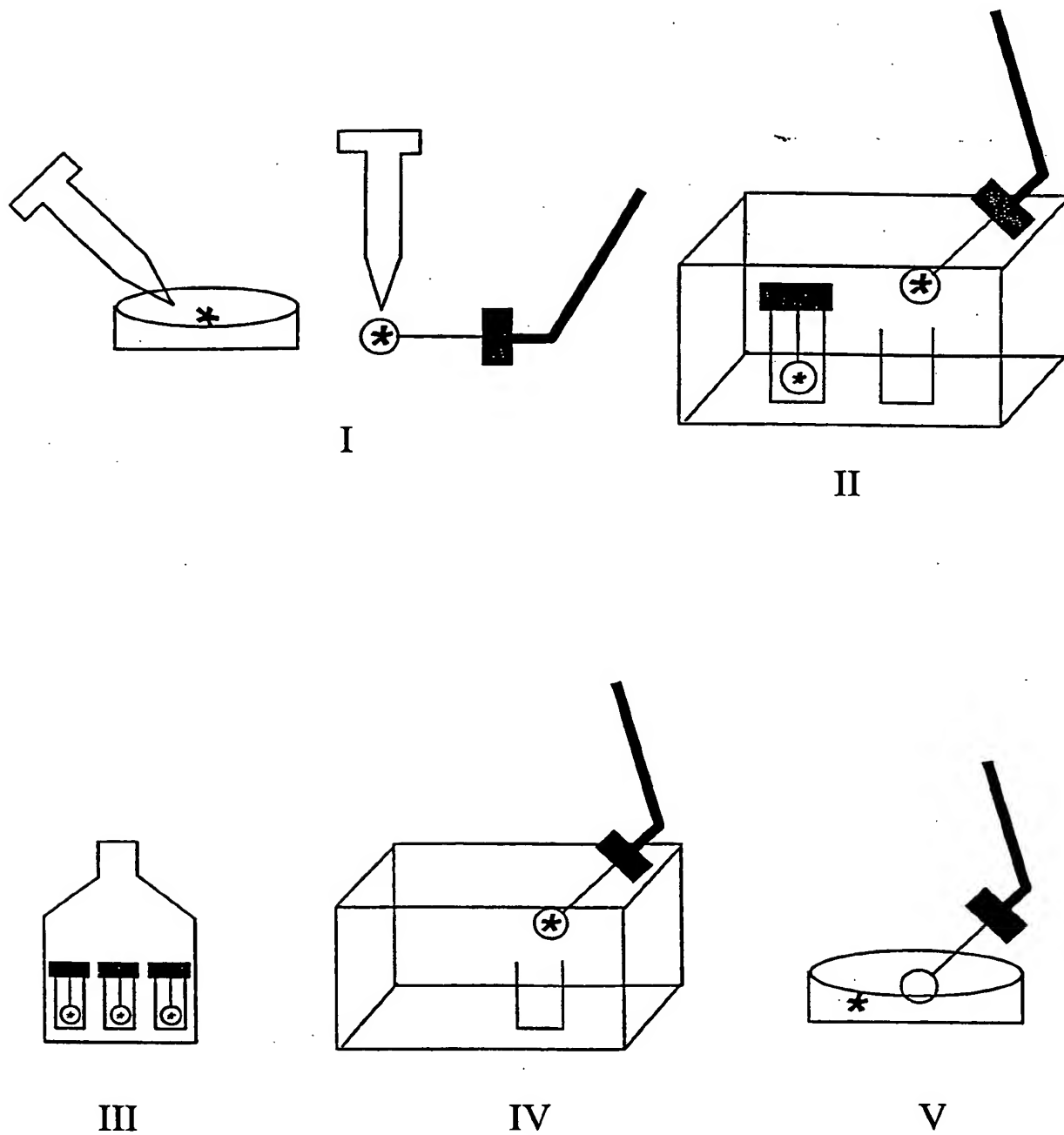


Fig. 1

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/23628**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) :A01N 1/02

US CL :435/1.3

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/1.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

US Pats, Biosis

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	Database Biosis, AN 1991:496738, RIHA et al. 'Vitrification of Cattle Embryos by Direct Dropping into Liquid Nitrogen and Embryo Survival after Nonsurgical Transfer' abstract, Zivocisna Vyroba, 36(2), 1991, pages 113-119.	16, 17, 20-25, 27, 28 ----- 1-32
X - Y	MARTINO et al. Development into Blastocysts of Bovine Oocytes Cryopreserved by Ultra-Rapid Cooling. Biology of Reproduction. 1996, Vol. 54, pages 1059-1069, see Materials and Methods.	1, 2, 4-8, 16, 17, 20, 21, 23-28 ----- 1-32

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 04 JANUARY 2000	Date of mailing of the international search report 02 FEB 2000
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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/23628

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	SZELL et al. Rapid Cryopreservation of Sheep Embryos by Direct Transfer into Liquid Nitrogen Vapour at -180C. Reproduction, Fertility and Development. 1990, Vol. 2, pages 613-618, see Materials and Methods.	1, 2, 4-9; 16-18, 20, 21, 23-25, 27, 28 ----- 1-32
X - Y	VAJTA et al. Vitrification of Porcine Embryos using the Open Pulled Straw (OPS) Method. Acta Veterinaria Scandinavica. 1997, Vol. 38, No. 4, pages 349-352, see Materials and Methods.	1, 2, 4-8, 16-18, 20, 21, 23-25, 27, 29, 30, 32 ----- 1-32

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/23628

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-32 directed to embryo vitrification

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/23628

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-32, drawn to methods of vitrification of embryos, sperm, blastocysts, morulae.

Group II, claims 33-34, drawn to a kit comprising a medium, instructions, a loop, a vial.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as follows:

embryo, sperm, oocyte, blastocyst, morula

The claims are deemed to correspond to the species listed above in the following manner:

embryo or blastocyst - claim 24

embryo - claim 29

The following independent claims are generic: 1, 18, 20, 24, 29

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The kit of Group II is not required for the practice of the invention of Group I. Therefore, it is not a special technical feature.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: The species are not linked by a special technical feature because each species is a distinct cell type with art recognized individual requirements with respect to cryopreservation.